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Minireview

MINIREVIEW

Chlamydia psittaci: update on an underestimated zoonotic agent

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One sentence summary: A concise overview of current knowledge on Chlamydia psittaci, its pathogenic features and strategies to deal with host defense.
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ABSTRACT

Chlamydia (C.) psittaci is an economically relevant pathogen in poultry and pet birds, where it causes psittacosis/ornithosis, and also a human pathogen causing atypical pneumonia after zoonotic transmission. Despite its well-documented prevalence, the agent has received less attention by researchers than other *Chlamydia* spp. in the last decades. In the present paper, we review recently published data on *C. psittaci* infection and attempt to single out characteristic features distinguishing it from related chlamydial agents. It is remarkable that *C. psittaci* is particularly efficient in disseminating in the host organism causing systemic disease, which occasionally can take a fulminant course.

At the cellular level, the pathogen's broad host cell spectrum (from epithelial cells to macrophages), its rapid entry and fast replication, proficient use of intracellular transport routes to mitochondria and the Golgi apparatus, the pronounced physical association of chlamydial inclusions with energy-providing cell compartments, as well as the subversive regulation of host cell survival during productive and persistent states facilitate the characteristic efficient growth and successful host-to-host spread of *C. psittaci*.

At the molecular level, the pathogen was shown to upregulate essential chlamydial genes when facing the host immune response. We hypothesize that this capacity, in concert with expression of specific effectors of the type III secretion system and efficient suppression of selected host defense signals, contributes to successful establishment of the infection in the host. Concerning the immunology of host–pathogen interactions, *C. psittaci* has been shown to distinguish itself by coping more efficiently than other chlamydiae with pro-inflammatory mediators during early host response, which can, to some extent, explain the effective evasion and adaptation strategies of this bacterium. We conclude that thorough analysis of the large number of whole-genome sequences already available will be essential to identify genetic markers of the species-specific features and trigger more in-depth studies in cellular and animal models to address such vital topics as treatment and vaccination.

Key words: *Chlamydia psittaci*; whole-genome analysis; epidemiology; animal models; host adaptation; immune evasion; immune response; molecular and clinical pathogenesis

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HISTORY AND EPIDEMIOLOGY

The obligate intracellular bacterium *Chlamydia (C.) psittaci*, the causative agent of human and avian psittacosis, is usually not ranked high on priority pathogen lists. Even among chlamydial organisms, it has largely remained in the shadow of the human pathogens *C. trachomatis* and *C. pneumoniae*, which attracted the main interest of researchers in the past three decades. This is in contrast to the perception in the 19th and early 20th centuries, when both psittacosis and its agent were in the focus of human and veterinary medicine.

The pioneering work in the 1960s and 1970s of J.W. Moulder, A. Matsumoto, G.P. Manire, T.P. Hatch, P.B. Wyrick and several other scientists, who used *C. psittaci* as the model organisms in their studies, laid the groundwork for modern chlamydia research. Moulder (1962) conducted the first molecular characterization of chlamydiae when analyzing the structure and chemical composition of *C. psittaci* 'particles'. The first high-resolution images of chlamydial bodies were obtained for *C. psittaci* by Matsumoto and Manire (1970) using electron microscopy, and they are still used as a reference by chlamydia researchers nowadays for their outstanding quality. Another groundbreaking work was accomplished by Hatch (1975), who demonstrated the requirement of *C. psittaci* for energy intermediates from the host cell. In 1978, Wyrick and colleagues first described the structural features of chlamydial compartments (Narita, Wyrick and Manire 1976) and showed the capability of *C. psittaci* to infect immune cells (Wyrick and Brownridge 1978). The first observation of 'persistent forms' was also made in *C. psittaci* (Moulder, Levy and Schulman 1980), as was the first isolation of a chlamydiaphage (Richmond, Stirling and Ashley 1982).

The first scientific article on a chlamydial infection referred to an outbreak of psittacosis in humans (Ritter 1879). In the period from 1890 to 1930, numerous severe outbreaks of human psittacosis occurred in Europe, North and South America (Hegler 1930; Meyer and Eddie 1935), all of which could be attributed to handling, sale and purchase of parrots and other exotic birds. In fact, the impact of these epizootic events can be best understood by recalling that the creation of the National Institutes of Health was contributed to by an outbreak of psittacosis in the United States in 1930 (Lepore 2009). Later on, large outbreaks became rare exceptions as disease control was more effective due to improved knowledge on etiology and epidemiology, better diagnostic tools, as well as the use of antimicrobials in therapy.

Nowadays, avian chlamydiosis (also referred to as psittacosis, ornithosis or parrot fever) is still widespread and represents a major factor of economic loss to the poultry industry (European Commission 2002), as well as a permanent risk for zoonotic transmission to man (Harkinezhad, Geens and Vanrompay 2009). The recent discovery of two more avian chlamydial species, i.e. *C. avium* and *C. gallinacea* (Sachse *et al.*, 2014), implies the likelihood of a more complex etiology of avian chlamydiosis. There are indications that, in certain cases, *C. psittaci* may act in concert with either of the new species (Krautwald-Junghanns *et al.*, 2013). Apart from overt clinical manifestations (see Section 'Clinical manifestations in humans and animals'), latent *C. psittaci* infection in birds can cause recurrent clinical disease and lead to chronicity. The mere carrier status of domestic poultry could also be economically relevant since, in cattle, retarded development and impaired performance in chlamydia-infected animals were observed even in the absence of clinical signs (Reinhold, Sachse and Kaltenboeck 2011). This means that adverse effects of *C. psittaci* infection on animal welfare should not be underestimated. In addition, intermittent shedding by carriers represents an important reservoir of infection for birds and humans.

Until 1999, the species *C. psittaci* (Moulder *et al.*, 1984) comprised four serovars representing strains from birds, ruminants, cats and guinea pigs, respectively. Based on ribosomal RNA sequence analysis, these serovars were reclassified to form separate species (Everett, Bush and Andersen 1999). The revised species *Chlamydophila psittaci*, meanwhile renamed as *Chlamydia psittaci* (Kuo *et al.*, 2011), had been intended to retain only the avian strains. However, this proved untenable because meanwhile the agent has been found frequently in non-avian domestic animals, such as cattle (Kemmerling *et al.*, 2009), sheep (Lenzko *et al.*, 2011), swine (Vanrompay, Geens and Desplanques 2004; Kauffold *et al.*, 2006), horses (Szeredi, Hotzel and Sachse 2005; Theegarten *et al.*, 2008), goats and cats (Pantchev *et al.*, 2010), as well as in wildlife (Hotzel *et al.*, 2004) and laboratory rodents (Henning *et al.*, 2008). The role of *C. psittaci* in non-avian hosts is not yet completely clear, but it seems certain that cases of clinical disease are rarely occurring, even though the agent was shown to be capable of inducing disease in experimentally infected calves (Reinhold *et al.*, 2012). Dual infections involving *C. psittaci* and *C. pecorum* or *C. abortus* (as well as the combination of *C. pecorum* and *C. abortus*) are quite common in mammals (Pantchev *et al.*, 2010; Lenzko *et al.*, 2011), thus raising the possibility of synergetic effects that could influence the course of infection. The opportunity of non-avian *C. psittaci* strains to cause infection in humans appears to be negligible compared to avian isolates, since reported cases of human psittacosis are usually traced back to contact with an avian source. While the prevalence of the agent in the human population is generally low, it was recently found to be higher than that of *C. pneumoniae* among patients of community-acquired pneumonia in Germany, i.e. 2.2% vs. 1.4% based on PCR data (R. Dumke *et al.*, [unpublished data]).

Whether the differential infectious potential of avian and non-avian strains corresponds to intrinsic differences in virulence is not known. However, genetic markers that reflect different host or disease specificity have not been found in comparative genomic studies (Voigt, Schöfl and Saluz 2012), nor have any robust virulence factors been identified (Read *et al.*, 2013). Considering these findings and practical experience, one can hypothesize that birds represent the original and typical host for *C. psittaci*, while mammals merely act as alternate, transient hosts. The possibility that any passage of an avian strain through a non-avian host could result in loss of virulence also exists.

GENETICS AND GENOMICS

In the pre-genomics era, the *ompA* locus was the first to be used for subtyping of *C. psittaci*. It encodes the major outer membrane protein (MOMP), a cysteine-containing surface antigen of ca. 40 kDa representing approximately 60% of the weight of the outer membrane. This molecule harbors several genus- and species-specific antigenic determinants in the conserved regions and serotype-specific epitopes in its four variable domains (Conlan, Clarke and Ward 1988). The serotypes were initially defined by monoclonal antisera, but were later shown to be equivalent to *ompA* genotypes (Vanrompay *et al.*, 1997), which led to serotyping being superseded by genotyping in the late 1990s. Until recently, nine genotypes (former serotypes) of *C. psittaci* were recognized and a certain degree of host preference was assigned to them (Andersen 1991, 1997; Vanrompay *et al.*, 1993;Geens *et al.*, 2005), i.e. genotype A occurring in

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psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, F in parakeets, WC in cattle and M56 in rodents. In practice, it is more realistic to refer to host predilection rather than host specificity of the genotypes in order to account for quite a few exceptions. Subsequently, six more ompA genotypes were identified in psittacine and wild birds, i.e. 1V, 6N, Mat116, R54, YP84 and CPX0308 (Sachse *et al.*, 2008).

While ompA genotyping can be useful in epidemiological investigations, it is being replaced with the more discriminating multi-locus sequence typing (MLST). The protocol by Pannekoek and colleagues (Pannekoek *et al.*, 2008) is currently mostly used for MLST of *C. psittaci*. It is based on discriminatory segments of the seven housekeeping genes *eno*A, *fum*C, *gat*A, *gid*A, *hem*N, *hlf*X and *opp*A. In accordance with generally agreed MLST principles (Maiden 2006), each of these loci displays a similar level of nucleotide sequence variation, is not located adjacent to genes encoding putative outer membrane, secreted or hypothetical proteins to exclude diversifying selection, and the loci are sufficiently far apart from each other on the chromosome. This system has revealed links between individual *C. psittaci* sequence types and host species (Pannekoek *et al.*, 2010). However, mammalian *C. psittaci* strains of genotype A clustered with strains isolated from psittacine birds (Pannekoek *et al.*, 2012), which again indicates the lack of genetic differences between avian and non-avian isolates at this level.

Comparative genomics among members of the family *Chlamydiaceae* is currently still at an early stage, but the studies conducted so far have already revealed a number of characteristic features. On the one hand, conserved synteny, i.e. sequence and gene order conservation, in a genome of reduced size is recognized as a hallmark of the genus *Chlamydia* (Myers, Crabtree and Huot 2012). This should be regarded in the context of evolutionary restrictions due to the obligate intracellular lifestyle, which imply dependence on host substrates and metabolic capabilities (Read *et al.*, 2000). The degree of conservation is illustrated in the pan-genome of *Chlamydiaceae*, which comprises roughly two-thirds of all chlamydial proteins. Thus, 736 protein-coding sequences (CDS) are shared among the species of *C. psittaci*, *C. abortus*, *C. pneumoniae* and *C. trachomatis*, with the total CDS count of these species ranging from 874 to 1097 (Voigt *et al.*, 2012). Analysis of 20 *C. psittaci* genomes revealed that a total of 911 core CDS are shared among all *C. psittaci* strains sequenced so far, which is equivalent to about 90% of the genes present in each of these genomes (Read *et al.*, 2013).

All sequenced *C. psittaci* strains possess a single chromosome of approximately 1.1 Mbp. In addition, a conserved 8 kbp plasmid harboring seven to eight CDS has been found in the majority of strains. Upon submission of this manuscript, the whole-genome sequences (WGS) of 48 *C. psittaci* strains were deposited at the NCBI Genome database at different states of assembly, while an additional 44 unassembled entries were available from the Sequence Read Archive (SRA). At least for the fully and partially assembled genomes, this is more than half the number of *C. trachomatis* genomes ($n = 88$) and considerably more than that for *C. pneumoniae* ($n = 6$). Fully assembled sequences were available for 17 different strains of *C. psittaci*. An overview of basic parameters of selected strains is given in Table 1. Despite the huge amount of raw sequence data, very few advanced studies on *C. psittaci* WGS have been conducted. Two comparative studies on *Chlamydiaceae* genomes included type strain 6BC (Voigt *et al.*, 2012; Sachse *et al.*, 2014), and only one study actually compared WGS of individual *C. psittaci* strains (Read *et al.*, 2013). This situation is in contrast to *C. trachomatis* and *C. pneumoniae*, where several such studies have been conducted [reviewed in Bachmann, Polkinghorne and Timms (2014)].

A significant amount of interspecies diversity can be encountered in the plasticity zone (PZ), a hypervariable region near the predicted replication termination region (Read *et al.*, 2000). Size and organization of the PZ differ substantially among *Chlamydiaceae*, ranging from 45 genes in *C. trachomatis* to 6 genes in koala strain LPCoLN of *C. pneumoniae* (Voigt *et al.*, 2012). In *C. psittaci* strain 6BC, the PZ is 29 kbp and encodes 16 genes. One of its most important features is the large cytotoxin/adhesin (*tox*) gene, which is similar to the EHEC adherence factor and clostridial cytotoxins (Belland *et al.*, 2001), as well as the three-gene *guaAB-add* cluster, which plays a role in salvaging biosynthesis of purine nucleotides required for chlamydial growth. The majority of strains examined by Read and colleagues were found to have intact *tox* gene and *guaAB-add* loci (Read *et al.*, 2013). Interestingly, these loci are not functional in the closely related species *C. abortus* (Thomson *et al.*, 2005).

Another source of diversity among *Chlamydia* spp. is the family of *pmp* genes. The polymorphic membrane proteins (Pmps) have an N-terminal signal sequence, a C-terminal autotransporter-like domain, a central 'passenger' domain including a varying number of the *Chlamydia*-specific short tetrapeptide motifs GGA(I, L, V) and FXXN on the N-proximal side as their main structural domains. However, the overall sequence similarity between individual Pmps is low. The *C. psittaci* genome was shown to harbor 21 *pmp* family members (Voigt *et al.*, 2012). In individual *pmp* genes, the numbers of conserved tetrapeptide motifs ranged from 2 to 18 for GGA(I, L, V), and from 4 to 23 for FXXN. Repetitive tetrapeptide units are suggested to play a role in chlamydial adhesion to host cells (Molleken, Schmidt and Hegemann 2010), but further studies are required to verify the postulated adhesin function of Pmps.

Like other Gram-negative bacteria, chlamydiae possess a type III secretion system (T3SS) (Hsia *et al.*, 1997). At the intracellular stage of the developmental cycle, which confines the bacteria to an encapsulated vacuole-like compartment, the pathogen uses this sophisticated molecular machinery for secretion of effectors to ensure its own survival, sustain its growth and development and contain or challenge the host response (see Section 'Clinical manifestations in humans and animals'). Depending on the search algorithm, Voigt and co-workers identified 40 or 35 putative effector molecules in the WGS of *C. psittaci* strain 6BC (Voigt *et al.*, 2012). As only a few of them have been functionally characterized, a lot of research is still required to fill this essential gap.

Among T3SS effectors, the Inc proteins are important representatives as they are involved in modification of the inclusion membrane to serve as the interface between the chlamydial parasitophorous vacuole and the host cell cytosol and organelles. In *C. psittaci*, three representatives, IncA, B and C, have been identified so far (vs. seven in *C. trachomatis*). Due to its high immunogenicity in guinea pigs, IncA of *C. caviae* was the first Inc protein to be discovered (Rockey, Heinzen and Hackstadt 1995). While the members of this protein family display little general sequence similarity, they share a characteristic bilobed hydrophobic domain of 60–80 amino acid residues. Interestingly, the number of APA or AGA tandem repeat sequence motifs in *incA* was recently found to associate highly significantly with virulence of *C. pecorum* strains (Mohamad *et al.*, 2014).

A similar inclusion membrane-associated function has been suggested for the transmembrane head proteins (TMHs), which are encoded by genes located in the TMH/Inc cluster (Thomson *et al.*, 2005). As these T3SS effectors possess paired N-terminal

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Table 1. Selection of *C. psittaci* WGSs*.

Strain	Host	Isolation (year, country)	Genotype (ompA)	Chromosome INSDC	Plasmid INSDC	Genome size (bp)	GC% (mol-%)	Scaffolds	Genes	Proteins	Reference
6BC	Psittacine	1983, USA/CA	A	CP002549.1	CP002550.1	1171 660	39.1	2	1016	975	Voigt *et al.* (2011)
6BC				CP002586.1	CP002587.1	1171 667	39.1	2	1055	1009	Grinblat-Huse *et al.* (2011)
6BC				SRA061584				SRA only	1297		Read *et al.* (2013)
C19/98	Sheep	1998, Germany	A	CP002804.1	–	1169 374	39.0	1	1019	978	Schofl *et al.* (2011)
01DC11	Swine	2001, Germany	A	CP002805.1	–	1172 197	39.1	1	1019	975	Schofl *et al.* (2011)
02DC15	Cattle	2002, Germany	A	CP002806.1	–	1172 182	39.1	1	1020	978	Schofl *et al.* (2011)
08DC60	Human	2008, Germany	A	CP002807.1	–	1172 032	39.1	1	1019	973	Schofl *et al.* (2011)
Mat116	Psittacine	2006, Japan	Mat116	CP002744.1	–	1163 362	39.1	1	1058	1017	Fukushi *et al.* (direct submission)
01DC12	Swine	2001, Germany	E	HF545614.1	HF545615.1	1171 011	39.1	2	1015	959	Seth-Smith *et al.* (2013)
RD1	Unknown	2010, UK	n.a.	FQ482149.1	FQ482150.1	1164 076	39.1	2	1009	951	Seth-Smith *et al.* (2011)
84/55	Psittacine	?, Germany	A	CP003790.1	CP003812.1	1172 064	39.1	2	1168	1127	Van Lent *et al.* (2012)
CP3	Pigeon	1958, USA/CA	B	CP003797.1	CP003813.1	1168 150	39.1	2	1165	1124	Van Lent *et al.* (2012)
CP3				SRA061582				SRA only	1058		Read *et al.* (2013)
GR9	Duck	1960, Germany	C	CP003791.1	–	1147 152	39.1	1	1083	1042	Van Lent *et al.* (2012)
GR9				SRA061587				SRA only	1052		Read *et al.* (2013)
CT1	Turkey	1954, USA/CA	C	SRA061680				SRA only	1063		Read *et al.* (2013)
NJ1	Turkey	1954, USA/NJ	D	CP003798.1	CP003816.1	1161 434	39.0	2	1093	1052	Van Lent *et al.* (2012)
NJ1				SRA061578				SRA only	1364		Read *et al.* (2013)
FalTex	Turkey	1980, USA/TX	D	SRA061585				SRA only	1332		Read *et al.* (2013)
Borg	Human	1944, USA/LA	D	SRA061576				SRA only	1159		Read *et al.* (2013)
MN	Human	1934, USA/CA	E	CP003792.1	CP003815.1	1168 490	39.1	2	1084	1041	Van Lent *et al.* (2012)
MN			E	SRA061581				SRA only	1386		Read *et al.* (2013)
VS225	Psittacine	1991, USA/TX	F	CP003793.1	CP003817.1	1157 385	39.0	2	1157	1116	Van Lent *et al.* (2012)
VS225				SRA061577				SRA only	1053		Read *et al.* (2013)
WS/RT/E30	Duck	2001, Germany	E/B	CP003794.1	CP003819.1	1140 789	39.0	2	1093	1052	Van Lent *et al.* (2012)
M56	Muskrat	1961, Canada	M56	CP003795.1	CP003814.1	1161 385	38.8	2	1090	1049	Van Lent *et al.* (2012)
M56				SRA061583				SRA only	1200		Read *et al.* (2013)
WC	Cow	1963, USA/CA	WC	CP003796.1	CP003818.1	1172 265	39.1	2	1095	1054	Van Lent *et al.* (2012)
WC				SRA061579				SRA only	1237		Read *et al.* (2013)
RTH	Hawk	2003, USA/CA	G	SRA061571				SRA only	1306		Read *et al.* (2013)

*Only strains with fully assembled WGS and/or those included in comparative studies are presented here. Another 30 partially assembled sequences can be found at http://www.ncbi.nlm.nih.gov/genome/?term = Chlamydia+psittaci.

SRA: Sequence Read Archive (www.ncbi.nlm.nih.gov/sra).

n.a.: not applicable

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transmembrane helices, paralogous to IncA, they are assumed to be members of an extended family of Inc proteins. In *C. psittaci*, the *tmh* locus encompasses eight genes, all of which carry the IncA domain (Voigt *et al.*, 2012).

The variable genomic regions discussed here presumably represent key factors involved in species-specific adaptation to host organisms and environmental niches, tissue tropism, as well as pathogenesis and virulence (Thomson *et al.*, 2005). Nevertheless, researchers are only beginning to discern the properties and capabilities distinguishing *C. psittaci* from other *Chlamydia* spp. All in all, specific genome-derived knowledge on *C. psittaci* is still very limited. Further in-depth analysis of the already generated WGS data is urgently needed to identify genomic parameters reflecting the peculiar features of this pathogen.

CLINICAL MANIFESTATIONS IN HUMANS AND ANIMALS

According to data from animal models, *C. psittaci* seems to be a particularly potent, flexible and resilient pathogen. In pulmonary infection of mice, the dose causing severe clinical signs was about 3 logs lower for *C. psittaci* (4×10^4 IFU per C57BL/6 mouse) than for *C. pneumoniae* in an otherwise identical setting (Sommer *et al.*, 2009; Bode *et al.*, 2012). Also in comparison with other *Chlamydia* spp., the agent was found to be more prolific in certain experimental settings. In chicken embryos, *C. psittaci* infection caused significantly higher mortality rates than the closely related *C. abortus*. The agent also clearly surpassed *C. abortus* by disseminating more extensively in host organs, eliciting higher macrophage numbers and causing upregulation of pro-inflammatory mediators (Braukmann *et al.*, 2012). In calves, different infectious doses of *C. psittaci* were shown to generate severe, moderate or subclinical respiratory symptoms (Reinhold *et al.*, 2012).

In cell culture, the average *C. psittaci* strain is distinguished from other *Chlamydia* spp. by rapid growth in a relatively short cycle period (48 h or less), high yield and relatively large inclusions. The success rate of recovering isolates from clinical tissue is comparatively high.

A simple explanation for differences in virulence across chlamydial species would refer to proliferation, i.e. the pathogen's ability to rapidly and efficiently reproduce in the host. Higher replication rates will result in a better ability to establish the infection by producing a given amount of viable EBs faster, and, thus, induce a more vigorous and potentially damaging inflammatory response in the host. Nevertheless, rapid growth is not a helpful criterion when virulence is compared along the spectrum of chlamydial pathogens, since, for instance, *C. muridarum* as the fastest grower is a natural commensal of the mouse. Likewise, a recent study on two variants of *C. psittaci* strain 6BC showed that attenuated and virulent strains do apparently not differ in growth kinetics *in vitro* and *in vivo* (Miyairi *et al.*, 2011). Instead, the length of the developmental cycle does affect the outcome of infection. This parameter is independent of the RB growth rate but depends on inclusion growth, which is itself dependent on the ability to acquire lipids.

Moreover, *C. psittaci* seems to modulate virulence by alteration of host immunity, which is assumed to be conferred by a remarkably small number of point mutations (SNPs) on the chromosome (Miyairi *et al.*, 2011). The same study singled out a homolog of the eukaryotic-like serine/threonine protein kinase gene *pkn5*, which encodes a putative effector protein of the chlamydial T3SS, as a candidate virulence gene. Sequence analysis revealed a non-synonymous mutation in the attenuated strain at a potential phosphorylation site near the C-terminus of Pkn5 that alters a conserved serine residue to glycine. The *pkn5* gene is part of an operon encoding a conserved T3SS (Peters *et al.*, 2007), and secretion of *C. trachomatis* Pkn5 via T3SS has been demonstrated in an orthologous *Salmonella enterica* serovar Typhimurium system (Ho and Starnbach 2005). Additionally, Pkn5 of *C. pneumoniae* has been shown to localize at the inclusion membrane and may directly interact with the host, thus serving as a potential virulence factor candidate (Herrmann *et al.*, 2006). While *C. trachomatis* Pkn5 (CT673), which lacks the activation domain I and the critical arginine residue in domain XI, seems to have lost kinase activity (Verma and Maurelli 2003), Herrmann and colleagues reported that Pkn5 homologs of *C. pneumoniae* (Cpn0703) and *C. psittaci* retained this particular arginine residue (Herrmann *et al.*, 2006).

Humans become infected through inhalation of aerosolized bacteria when exposed to infected birds or handling contaminated feathers, fecal material or carcasses (West 2011). Even transient exposure to infected birds and/or a contaminated environment can result in human infection.

The incubation period of *C. psittaci* in humans is 5–14 days (CDC 2000). Common symptoms of psittacosis include abrupt onset of fever, chills, headache, malaise, myalgia, nonproductive coughing and dyspnea. Other complications include pericarditis, endocarditis or myocarditis, hepatomegaly and splenomegaly. Fatal cases have become extremely rare (around 5%) since the advent of antibiotics (CDC 2000; West 2011). However, if the first upper respiratory signs associated with psittacosis are not treated, severe disease or even death may result. Late recognition of the disease is a real possibility even nowadays, because *C. psittaci* is not part of the routine diagnostic schedule in most medical laboratories (Senn and Greub 2008).

The respiratory tract does not seem to be the only tissue targeted by *C. psittaci*. In a recent study in trachoma patients, 19.0% of the cases were shown to include *C. psittaci*, either alone or in dual infection, mainly with *C. trachomatis*. This indicates that, in concert with the established causative agent *C. trachomatis*, *C. psittaci* may play a role in human trachoma (Dean *et al.*, 2013).

Since *Chlamydia* spp. are known to be mitogenic *in vitro* (Byrne and Ojcius 2004), cause resistance to apoptosis in infected cells (Miyairi and Byrne 2006) and induce polyclonal cell proliferations *in vivo* (Ferreri, Ernberg and Copie-Bergman 2009), it is not surprising that *C. psittaci* and other chlamydial agents are also discussed to be associated with cancerous diseases. Indeed, the microorganism was identified in ocular adnexal MALT (mucosa-associated lymphoid tissue) lymphomas (OAMLs) (Ferreri *et al.*, 2004; de Cremoux *et al.*, 2006). While MALT lymphomas are diverse in terms of clinical manifestation, they often coincide with bacterial infections, typically leading to specific gene deregulation (Collina *et al.*, 2012). Patients with OAML were reported to have a high prevalence of *C. psittaci* infection in both tumor tissue (Aigelsreiter *et al.*, 2011) and peripheral blood mononuclear cells (Ferreri *et al.*, 2004). *Chlamydia psittaci* seems to be present as a viable bacterium within the lymphomatous tumors (Ponzoni *et al.*, 2008), preferentially occurring in external antigen-exposed organs, where monocytes/ macrophages could act as main reservoirs for the pathogen. It has been speculated that persistent *C. psittaci* infections (see Section 'Intracellular persistence') might play a key role in OAML development, which is supported by the fact that *C. psittaci* suppression via doxycycline is accompanied by detectable lymphoma regression (Ferreri *et al.*, 2005). The prevalence of *C. psittaci* infection in MALT

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lymphomas seems to vary among regions (Chanudet *et al.*, 2006), being most frequent in Germany (47%), the East Coast of the USA (35%) and the Netherlands (29%). Clearly, further studies are required to demonstrate a causal link between the occurrence of *C. psittaci* in affected tissues and development of MALT lymphomas.

Avian *C. psittaci* infections are often systemic and can display unapparent, severe, acute or chronic manifestations (Andersen and Vanrompay 2000; Kaleta and Taday 2003). In birds, the bacteria infect mucosal epithelial cells as well as macrophages of the respiratory tract. Sepsis eventually develops and *C. psittaci* localizes in cells of conjunctiva, the gastrointestinal tract and most organs (Stewardson and Grayson 2010). Depending on the chlamydial strain and the avian host involved, the infection leads to pneumonia, air sacculitis, pericarditis, hepatitis and/or splenitis, occasionally with fatal outcome. Also here, it is thought that the bacteria use blood monocytes/macrophages as a vehicle to disseminate through the host organism (Beeckman and Vanrompay 2010).

Recent studies addressed pulmonary lesions in calves and mice aerogenously infected with *C. psittaci* to analyze species-specific reaction patterns in mammals (Reinhold *et al.*, 2012; Fiegl *et al.*, 2013; Knittler *et al.*, 2014). Based on the morphological findings, the authors initially observed infection of alveolar epithelial cells. Multiplication of the pathogen was followed by a rapid influx of neutrophil granulocytes, most likely mediated by cytokines released from infected cells. It was suggested by Knittler and co-workers (Knittler *et al.*, 2014) that degranulation and decay of neutrophil granulocytes cause extensive species-specific damage of the pulmonary tissue. Subsequently, the adaptive immune response, which involves dendritic cells (DCs), T and B lymphocytes, would accomplish (partial or complete) elimination of bacteria and finally pave the way for regeneration of pulmonary tissue.

HOST–PATHOGEN INTERACTION

The early stage

Elementary bodies (EBs) of *C. psittaci* are thought to infect their target cells in the lung via attachment to the base of cell surface microvilli (Beeckman *et al.*, 2008), where they are actively engulfed by endo- or phagocytic vesicles (Dautry-Varsat, Balana and Wyplosz 2004). Our own unpublished experimental observation that *C. psittaci* can enter almost any cell type (epithelial cells, fibroblasts, macrophages, DCs, etc.) suggests multiple modes of bacterial entry and can provide an explanation for the development of systemic *C. psittaci* infection in different host organisms. Studies on the *C. psittaci*-related species of *C. caviae* showed that initial attachment is mediated by electrostatic interactions, most likely with glycosaminoglycan (GAG) moieties on the host cell surface (Gutierrez-Martin *et al.*, 1997) (Fig. 1). However, the observation that cellular binding of *C. psittaci* and related chlamydial strains is only partially or not inhibited by heparin strongly suggests that further adherence mechanisms contribute to chlamydial attachment (Gutierrez-Martin *et al.*, 1997; Ojcius *et al.*, 1998). It was speculated that chlamydial cell contact is a two-step process, i.e. reversible binding followed by irreversible attachment (Carabeo and Hackstadt 2001). Although chlamydial entry is extremely efficient, the exact molecular details of bacterial uptake are not well understood. The host protein disulfide isomerase (PDI) has been identified as being essential for both *C. psittaci* attachment and entry into cells (Abromaitis and Stephens 2009) (Fig. 1). PDI is highly enriched in the endoplasmic reticulum, but is also found on the cell surface where it catalyzes reduction, oxidation and isomerization of disulfide bonds. Although cell surface PDI is necessary for *C. psittaci* attachment, the bacteria apparently do not bind directly to cell-associated PDI. Instead, disulfide reduction seems to be required for chlamydial entry, with bacterial attachment being independent of PDI enzymatic activity. It was demonstrated that attached *C. psittaci* is internalized very rapidly within 30–60 min (at 37°C). Electron microscopy data also suggest different cellular mechanisms for the chlamydial entry process. One hypothesis involves microfilament-dependent phagocytosis including contact between bacterial adhesins and host cell receptors (Ward and Salari 1982; Finlay and Cossart 1997), whereas another proposed uptake mechanism is based on receptor/clathrin-mediated endocytosis (Hodinka *et al.*, 1988), which is normally employed for cellular uptake of large biomolecules. Further studies provided evidence that some chlamydial strains might also enter the host cell using cholesterol-rich lipid raft domains (Norkin, Wolfrom and Stuart 2001; Stuart, Webley and Norkin 2003). Various chlamydial surface molecules have been proposed to function as adhesins during the attachment/uptake process of chlamydiae, e.g. the MOMP OmcB (Omp2) and GAGs (Ting *et al.*, 1995; Gutierrez-Martin *et al.*, 1997; Escalante-Ochoa, Ducatelle and Haesebrouck 1998; Moelleken and Hegemann 2008). Although EBs are larger than clathrin-coated vesicles (200–300 vs. 100–200 μM), *C. psittaci* appears to be capable of exploiting this cellular entry pathway (Hodinka and Wyrick 1986). It was suggested long ago that, depending on the chlamydial strain, host cell type and other cell biological conditions, chlamydiae are able to use different routes to enter their target cells (Wyrick *et al.*, 1989). Indeed, both clathrin-coated and non-coated vesicles were shown to facilitate chlamydial entry into polarized human endometrial cells (Wyrick *et al.*, 1989).

Several studies also highlighted the critical importance of host microfilaments, microtubules and microtubule motor proteins (kinesin and dynein) for uptake and intracellular development of *C. psittaci* and other *Chlamydia* spp. (Escalante-Ochoa, Ducatelle and Haesebrouck 2000; Carabeo *et al.*, 2002; Grieshaber, Grieshaber and Hackstadt 2003). In all cell types tested so far, participation of actin and tubulin seems to be necessary for optimal bacterial proliferation (Escalante-Ochoa *et al.*, 2000). On the other hand, the shutdown of prokaryotic protein synthesis seemed to have no effect on *C. psittaci* uptake, thus demonstrating that the internalization process does not require protein synthesis on the bacterial side (Friis 1972; Tribby, Friis and Moulder 1973; Jutras *et al.*, 2004). The uptake into the host cell rather depends on the energy-consuming function of a preformed macromolecular apparatus, i.e. the T3SS or injectisome (see also Section 'Genetics and genomics'), which enables the microorganism to arrange export of effector proteins into the cytosol and to the inclusion membrane, where they interact with host proteins and cause modulation of host cell functions (Hsia *et al.*, 1997; Scidmore 2011). As it is assumed that the chlamydial T3SS is kept active during the intracellular stage (Dautry-Varsat *et al.*, 2004) (Fig. 1), interactions of these effectors with host proteins seem to play a role from adhesion and internalization of EBs to their release from the host cell (Scidmore 2011). Like in other Gram-negative bacterial pathogens, the macromolecular T3SS complex of chlamydiae spans the inner and outer membranes as well as the plasma membrane (as extracellular bacteria are internalized by the host cell), or the inclusion membrane (in intracellular bacteria during replication) (Plano, Day and Ferracci 2001). This kind of secretion system also allows for

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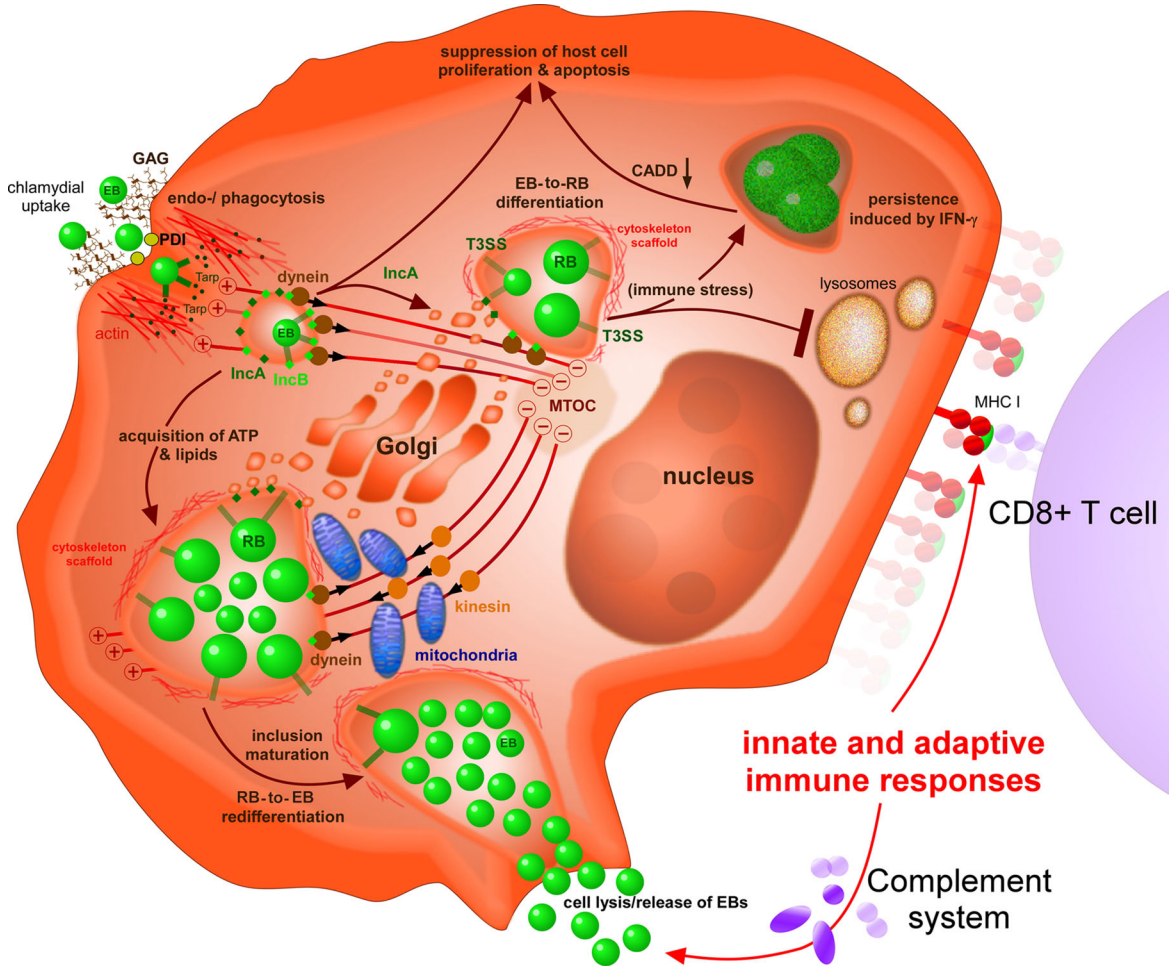

Figure 1. Developmental cycle of *C. psittaci*. Like other chlamydial species, *C. psittaci* is characterized by a biphasic developmental cycle that includes an intracellular stage in vacuole-like inclusion. Metabolically inactive but infectious EBs are taken up by the host cell. After internalization, EBs are surrounded by an intracellular membrane to form an inclusion. Inside this vesicle, EBs transform into larger, metabolically active RBs, which divide by binary fission. *Chlamydia psittaci* exploits the host's intracellular trafficking machinery by recruiting the microtubule motor protein dynein to the outer surface of the vacuole, which drives the migration of inclusions toward the minus end of microtubules and the microtubule-organizing center, where it resides in a central perinuclear position to establish its intracellular niche near the GA. Fusion, maturation and maintenance of chlamydial inclusions require clustering around the perinuclear region. During the maturation of inclusions, chlamydial effector proteins cause Golgi fragmentation and thereby ensure lipid acquisition and bacterial growth. Within 24–72 h, RBs transform back into infective EBs, which are subsequently released from inclusions to infect neighboring cells. In the presence of growth inhibitors, such as IFN-γ, intracellular chlamydiae can develop into a non-replicating, persistent form. During the intracellular stage, the *C. psittaci*-infected cells process bacterial proteins for antigen presentation. To this end, bacterial protein fragments (peptides) bind to MHC I molecules and are displayed on the cell surface. These are recognized by cytotoxic CD8+ T cells, which then destroy the infected host cell together with the pathogen. Released EBs of *C. psittaci* activate the complement cascade leading to the cleavage of inactive complement factors, which then stimulate different effector cells of the adaptive immune system via the respective complement receptor.

translocation into the host cell of bacterial proteins from an extracellular location across the bacterial cell envelope (Betts-Hampikian and Fields 2010), as well as for secretion of pre-synthesized proteins from the cell-attached EBs (Fields *et al.*, 2003; Jutras *et al.*, 2004; Jamison and Hackstadt 2008) (Fig. 1). In this context, it was pointed out that EBs become activated by a yet unknown signal triggering the T3SS during cell surface attachment (Jutras *et al.*, 2004).

Beeckman and co-workers provided evidence for the T3SS of *C. psittaci* assisting in the establishment of an optimal environment for intracellular bacterial growth (Beeckman *et al.*, 2008). Their experimental studies revealed that the essential structural T3SS protein SctW is associated with the bacterium and the inclusion membrane, while the T3SS proteins SctC and SctN are localized at the bacterium itself. Gene expression analysis demonstrated transcription of T3SS structural protein-encoding genes from mid-cycle onwards (12–18 hpi), whereas the genes encoding effector proteins and putative T3SS-related proteins are expressed early (1.5–8 hpi) or late (>24 hpi) in the developmental cycle. More recent studies in *C. psittaci*-infected human macrophages confirmed that the T3SS is continuously expressed and active throughout the whole infection cycle (Saad 2011). Moreover, it seems that newly formed EBs carry a preloaded T3SS in order to ensure rapid entry and subversion of new host cells.

When combined with findings on other *Chlamydia* spp., these data are exciting and could potentially provide clues for the elucidation of specific pathogenicity mechanisms. In particular, the action of T3SS effectors could be a significant factor underlying the distinctive properties of *C. psittaci* in terms of fitness, dissemination and evasion of the host immune system. However, realistically, we are only beginning to understand the role and significance of the T3SS in the wider context of *C. psittaci* infection.

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Intracellular survival of the pathogen

Once internalized in the early inclusion, the infecting EB transforms into a larger and more conventional bacterial form, the reticulate body (RB). Subsequently, the RB-containing inclusions translocate through a cytoskeleton-dependent mechanism to the perinuclear region, and RBs replicate by binary fission. Through unknown signals, the RBs re-transform into EBs after 24–72 h and are finally released from the host cell to infect adjacent cells or be transferred to new hosts (Fig. 1). Immediately after inclusion formation, the properties of the nascent bacterial compartment are modified by processes that are dependent on early chlamydial gene expression and active protein synthesis, which results in avoidance of lysosomal fusion (Scidmore, Fischer and Hackstadt 2003) (Fig. 1) and microtubule-dependent trafficking of the inclusion to the microtubule organizing center (MTOC) in the perinuclear region (Grieshaber *et al.*, 2003) (Fig. 1). *Chlamydia psittaci*-mediated prevention of lysosomal fusion is not due to a general repression of lysosome function (Eissenberg and Wyrick 1981; Ojcius *et al.*, 1998). Instead, the following two-stage mechanism controlling the intracellular escape of chlamydial compartments from lysosomal breakdown was suggested: (i) an initial phase of delayed lysosome maturation due to intrinsic properties of EBs and (ii) an active modification of vesicular interactions of the inclusion (Scidmore *et al.*, 2003). Moreover, chlamydial inclusions are known to intercept vesicular and non-vesicular pathways to obtain host-derived lipids, such as sphingomyelin, cholesterol, glycerophospholipids and neutral lipids (Hackstadt, Scidmore and Rockey 1995; Heuer *et al.*, 2009; Elwell and Engel 2012).

The mechanisms by which *C. psittaci* manages its intracellular survival are still under intensive investigation (Knittler *et al.*, 2014). Recent studies indicate that Inc proteins (Valdivia 2008) (see Section 'Genetics and genomics') are key players targeting different cellular pathways of the infected host (Borth *et al.*, 2011; Böcker *et al.*, 2014). Incorporation of Inc proteins in the inclusion membrane is mediated by the T3SS (Mital *et al.*, 2013). In *C. psittaci*, type III secretion of both IncA and IncB and incorporation in the inclusion membrane was experimentally demonstrated (Beeckman *et al.*, 2008). While their hydrophobic domain enables the anchoring of Inc proteins in the inclusion membrane, the cytoplasmic tail is responsible for interaction with host proteins (Jutras *et al.*, 2004). By virtue of the latter, Inc proteins could be regarded as central regulators of pathogen–host interactions as this interplay may affect different cell functions including signaling and trafficking (Mital *et al.*, 2013). In all chlamydial IncA proteins identified so far, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-like motifs have been found (Delevoye *et al.*, 2004). These motifs allow interactions with several host SNARE proteins, which are essential for membrane fusion (Delevoye *et al.*, 2008; Paumet *et al.*, 2009). Indeed, several host proteins have been identified as interaction partners for Inc proteins. For instance, two recent studies on *C. psittaci* described the host cell protein G3BP1 and components of the dynein complex (dynein motor proteins) as cellular interaction partners of IncA and IncB, respectively (Borth *et al.*, 2011; Böcker *et al.*, 2014) (Fig. 1). The IncA-binding partner G3BP1 harbors a phosphorylation-dependent RNase activity that specifically cleaves the 3′-untranslated region of human c-myc mRNA (Gallouzi *et al.*, 1998). Experimental evidence suggests that the interaction between chlamydial IncA and host G3BP1 affects c-myc expression and in turn suppresses cellular proliferation and host cell apoptosis (Borth *et al.*, 2011). The IncB-protein of *C. psittaci* seems to utilize dynein motor proteins (Roberts *et al.*, 2013) for controlling intracellular transport and perinuclear MTOC localization of inclusions in order to support bacterial growth in infected cells (Böcker *et al.*, 2014). The data of Böcker and co-workers (Böcker *et al.*, 2014) provide experimental evidence that the host protein Snapin forms a heterooligomeric complex with IncB and dynein and thereby physically connects *C. psittaci* inclusions with the microtubule network in infected cells (Fig. 1).

Chlamydiae are known to target actin, microtubules and intermediate filaments to regulate diverse aspects of their intracellular survival (Scidmore 2011) (Fig. 1). For example, the bacteria surround their inclusions with a mesh of host cytoskeletal filaments, which serve as a scaffold structurally stabilizing the bacterial compartment (Scidmore 2011) (Fig. 1). As a consequence, the activation of host cell defense mechanisms is limited because leakage of inclusion contents into the cytosol is prevented (Kumar and Valdivia 2008). Although participation of the actin and tubulin, as well as kinesin and dynein, is essentially required for optimal growth of *C. psittaci* (Escalante-Ochoa *et al.*, 2000), the functional involvement of cytoskeletal components in chlamydial development is still poorly understood.

Among cell organelles, mitochondria were found to be particularly closely associated with *C. psittaci* inclusions (Matsumoto 1981; Knittler *et al.*, 2014). Such an intimate association is likely to influence chlamydial development, since it is related to the acquisition of eukaryotic ATP (Fig. 1). Notably, this seems to be a characteristic feature of *C. psittaci* as nothing comparable has been observed for other chlamydial species, such as *C. trachomatis* or *C. pneumoniae* (Matsumoto *et al.*, 1991). The association appears so tight that mitochondria remain attached to the inclusion membrane even after isolation of inclusions from infected cells. While the functional significance of mitochondrial recruitment is not known, close attachment of the inclusion to mitochondria may enable *C. psittaci* to acquire host ATP and/or other high-energy storage substrates using its own alternate mechanism. Interestingly, the microtubule motor proteins kinesin and dynein are known to be associated with both organelles (Ball and Singer 1982; Brady and Pfister 1991). It seems that kinesin acts through the apposition of mitochondria to *C. psittaci* inclusions, whereas dynein is responsible for transport and placement of the inclusion in the perinuclear Golgi apparatus (GA) region (Escalante-Ochoa *et al.*, 2000; Böcker *et al.*, 2014) (Fig. 1).

The chlamydial T3SS effector translocated actin-recruiting phosphoprotein (Tarp), which is involved in chlamydial entry and survival (Engel 2004), is also found in *C. psittaci* (Beeckman *et al.*, 2008) (Fig. 1). Transcription of the Tarp-encoding gene occurs late during the developmental cycle (Beeckman *et al.*, 2008). Chlamydial EBs translocate pre-synthesized stored Tarp into the host cell and thereby facilitate an active actin remodeling process that results in reorganization of the cell surface (Engel 2004). It seems that Tarp, which contains several actin-binding domains (Jewett *et al.*, 2010), nucleates actin polymerization through direct interaction with actin (Jewett *et al.*, 2006).

Following chlamydial entry into cells, assisted by Tarp, chlamydia-containing vacuoles are transported to a perinuclear location in close proximity to the GA (Knittler *et al.*, 2014) (Fig. 1). Transport of chlamydiae to the MTOC requires host cell vesicle transport and is dynein dependent (Grieshaber *et al.*, 2003). Intracellular development of the inclusion is accompanied by extensive lipid acquisition from various sources

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(Mehlitz and Rudel 2013). One of the major lipid sources appears to be the GA (Hackstadt *et al.*, 1996), which is fragmented during chlamydial infection (Heuer *et al.*, 2009), probably to facilitate lipid transport to the inclusion. GA fragmentation has been observed for *C. trachomatis* as well as for *C. psittaci*, where the effect was found to be more pronounced (Knittler *et al.*, 2014). In the case of *C. psittaci*, infected cells display small GA fragments scattered throughout the whole cytosol (Knittler *et al.*, 2014). The use of fluorescently labeled ceramide, which normally undergoes conversion to sphingomyelin in early GA compartments, facilitated visualization of sphingomyelin incorporation in both inclusion membrane and RB cell wall (Jutras *et al.*, 2004). Several eukaryotic glycerophospholipids, such as phosphatidylinositol and phosphatidylcholine, were also found to be trafficked to the chlamydial inclusion (Jutras *et al.*, 2004).

Following the asynchronous RB-to-EB differentiation, cell lysis results in the release of a mixture of RBs and EBs (Moulder 1991). So far, the signal responsible for cell lysis and chlamydial release has not been identified. However, it is tempting to speculate that it involves host cell apoptosis induced by distinct chlamydial effector proteins (Jutras *et al.*, 2004).

Intracellular persistence

Chlamydial persistence usually denominates a state of infection when the pathogen remains viable but non-cultivable, while the host immune system is unable to eliminate it. Morphologically, this reversible state is characterized by aberrant bodies, i.e. enlarged pleomorphic RBs, and reduced inclusion size. To avoid confusion, the term 'aberrant RB phenotype' rather than 'persistence' has been proposed to refer to the phenomenon defined *in vitro* (Wyrick 2010; Bavoil 2014).

The phenomenon of *in vitro* persistence had already been observed in the 1960s, but was first addressed in a systematic manner in the 1980s. Again, the first experiments were conducted with *C. psittaci*. Moulder and colleagues (Moulder *et al.*, 1980) reported a subpopulation of murine fibroblasts (L cells) being infected with 'cryptic forms' of *C. psittaci*, which grew poorly and became resistant to superinfection. Later on, Beatty and colleagues (Beatty, Byrne and Morrison 1994) developed a physiologically relevant cell culture model of *C. trachomatis* persistence induced by IFN-γ that was based on tryptophan depletion (see Fig. 1). Meanwhile, a large number of other *in vitro* persistence-inducing factors have been described, e.g. amino acid deficiency (Coles *et al.*, 1993), iron depletion (Raulston 1997), exposure to antibiotics (Pantoja *et al.*, 2001), phage infection (Hsia *et al.*, 2000), co-infection with virus (Borel *et al.*, 2010) or a continuous infection model (Kutlin *et al.*, 2001).

The various *in vitro* persistence models share loss of infectivity and the appearance of small inclusions containing fewer bacteria, which are, however, larger than normal RBs (Morrison 2003; Wyrick 2010). The morphological characteristics also comprise the arrest of the chlamydial developmental cycle, a large reduction in the infectious titer, and, in some cases, increased 'resistance' to antibiotics. While *in vitro* persistence has been characterized extensively (Wyrick 2010), there have also been reports from *in vivo* experiments showing that chlamydiae were stressed during infection (Pospischil *et al.*, 2009; Phillips Campbell *et al.*, 2012). While these observations are interesting, they do not establish a cause–effect relationship between aberrant bodies and chronic infection and, therefore, are not evidence of chlamydial persistence *in vivo*. In fact, the true state of *in vivo* persistence might not be directly related to the various model systems used. It is hypothesized that recurrent chlamydial disease may result from persistence of organisms after unresolved infections (Hogan *et al.*, 2004).

Microscopic and cell biological studies showed that *C. psittaci* is also capable of entering a persistent state *in vitro*, which could conceivably play a role in chronic infections, as well as in failure of antibiotic therapy and immunoprophylaxis. Goellner and colleagues used three different *in vitro* persistence models (iron depletion, antibiotic treatment and IFN-γ exposure) to analyze morphological alterations and changes in mRNA transcription of *C. psittaci* (Goellner *et al.*, 2006). While the phenotypical characteristics were the same as in other chlamydiae, i.e. aberrant morphology of RBs, loss of cultivability and rescue of infectivity upon removal of inducers, the transcriptional response of *C. psittaci* to persistence-inducing factors provided several distinctive features. Consistent downregulation of genes coding for membrane proteins, transcription regulators, cell division factors and EB-RB differentiation factors from 24 hpi onwards proved to be a general feature of *C. psittaci* persistence. Other genes displayed considerable variations in response patterns from one model to another, which implies that there is no persistence model *per se*. In contrast to the results obtained for *C. trachomatis*, late shutdown of essential genes in *C. psittaci* was much more comprehensive with IFN-γ-induced persistence, which is probably due to the absence of a functional tryptophan synthesis operon (Goellner *et al.*, 2006). Most interestingly, the *C. psittaci* chlamydia protein associating with death domains (CADD) gene was found to be downregulated at 48 hpi in the presence of IFN-γ. This is in contrast to data from IFN-γ-induced persistence of *C. trachomatis*, where it was upregulated at 48 hpi (Belland *et al.*, 2003). CADD shares homology with the death domains of tumor necrosis factor family receptors and induces apoptosis when transiently transfected to non-infected cells (Stenner-Liewen *et al.*, 2002). Although it is not known at present whether CADD is the dominant protein governing apoptosis, a recent study demonstrated its capability to induce cell death, thus supporting the notion that apoptosis inhibition could be an integral part of *in vitro* persistent infections (Schwarzenbacher *et al.*, 2004) (Fig. 1). Whether and how this relates to actual latent, persistent or chronic infections that occur in humans and animals has, however, not been established.

NEW INSIGHTS INTO INNATE AND ADAPTIVE IMMUNE RESPONSE

The innate immunity is of key importance in primary recognition of chlamydial infections (Knittler *et al.*, 2014) (Fig. 1). There has been some evidence that *C. psittaci* can cope more efficiently than other chlamydiae with the increased release of pro-inflammatory mediators during early host response in chicken embryos (Braukmann *et al.*, 2012). These findings have been corroborated in young chicks (I. Kalmar *et al.*, [unpublished data]). Upregulation of chlamydial *incA* (probably contributing to stabilization of the inclusion), *ftsW* (chlamydial replication), *groEL* (chaperone co-localizing with macrophages) and *cpaf* (processing of host proteins controlling inclusion integrity (Bednar *et al.*, 2011; Bavoil and Byrne 2014; Snavely *et al.*, 2014) in a chicken model seems to reflect the specific capacities of *C. psittaci* in establishing the infection and disseminating in the host organism.

Furthermore, it has long been known that EBs activate the complement system *in vitro* thereby reducing their infectivity in cell culture (Fedorko *et al.*, 1987). The complement system consists of about 40 serum factors and is activated by components

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of pathogen surfaces (Klos *et al.*, 2013). It also modulates inflammatory responses, protects against extracellular pathogens and is often regarded as a key factor of the innate immunity. In a murine model of intranasal *C. psittaci*-lung infection, early, high and long-lasting activation of the complement system was observed (Bode *et al.*, 2012). The protective function of the complement cascade against *C. psittaci* seems to rely mainly on the anaphylatoxic peptide C3a and its receptor (C3a/C3aR). Indeed, recent experiments with C3aR$^{-/-}$ mice revealed that C3aR was indispensable for effective protection and enhanced survival in *C. psittaci* infection (Dutow *et al.*, 2014). Based on the experimental data, it was concluded that EBs activate the complement cascade, leading to cleavage of inactive complement factor C3 into active C3a and C3b. Bound to its antigen, the C3b derivative C3d stimulates B cells as well as antibody production of plasma cells via complement receptor 2. The C3aR is expressed on mature DCs and, most likely, also on different subtypes of activated T lymphocytes. It was hypothesized that C3a modulates the function of CD4+, CD8+ and/or regulatory T cells for development of an effective adaptive protection against *C. psittaci*. Moreover, C3a/C3aR can also activate DCs, thereby facilitating their enhanced migration to draining lymph nodes and augmented presentation of chlamydial antigens to CD4$^+$ and CD8$^+$ T cells (Knittler *et al.*, 2014). Indeed, consistent with the intracellular localization of chlamydiae, cell-mediated immune responses against *C. psittaci* and other chlamydial species have been observed in infected humans and mice (Lammert 1982; Starnbach, Bevan and Lampe 1995). Transfer of either CD4$^+$ and/or CD8$^+$ chlamydia-specific T cells into naïve mice was shown to protect the animals against challenge with chlamydiae (Starnbach, Bevan and Lampe 1994; Su and Caldwell 1995), and studies with MHC-deficient mice confirmed the importance of T cell-dependent responses (Beatty and Stephens 1994; Morrison, Feilzer and Tumas 1995).

DCs translate innate into adaptive immunity and are among the first professional antigen-presenting cells (pAPCs) encountered by chlamydiae in the course of infection (Gervassi *et al.*, 2004), and cytotoxic CD8$^+$ T cells, primed by infected DCs, likely play an important role in the effective anti-chlamydial immune response (Ojcius *et al.*, 1998; Gervassi *et al.*, 2004). It was recently demonstrated that *C. psittaci*-infected murine DCs use autophagosomal and endovacuolar processing for degradation of bacterial compartments, as well as proteolytic production of chlamydial peptide antigens (Fiegl *et al.*, 2013). These findings could have important implications for the future design of vaccination strategies based on DC-targeting antigens. The advantage of autophagosomal processing is that polypeptides from intracellular bacteria, which reside in inclusions and avoid lysosomal fusion, can still be generated for major histocompatibility complex (MHC) class I-mediated antigen presentation (Fiegl *et al.*, 2013) (Fig. 1). As clearance of chlamydiae depends on the ability of CD8+ T cells to recognize epithelial cells (Fig. 1), in which the bacteria predominantly replicate (Balsara and Starnbach 2007), one could imagine that, in *C. psittaci*-infected epithelial cells, IFN-γ from activated T cells creates a situation reflecting the above-described scenario for infected DCs. Indeed, in infected epithelial cells, IFN-γ was seen to restrict chlamydial growth (Morrison 2003), affect cytoplasmic translocation of chlamydial effectors (Heuer *et al.*, 2003), enhance MHC I expression and surface expression (Kagebein *et al.*, 2014), and induce autophagic degradation of chlamydiae (Al-Zeer *et al.*, 2009).

The development of anti-chlamydial T cell vaccines is the current focus of many research groups (Karunakaran *et al.*, 2010; Howie *et al.*, 2011; Picard *et al.*, 2012). Although effective antimicrobial therapies exist, vaccination is still considered the best approach to reduce the prevalence of chlamydial infections (Longbottom and Livingstone 2006; Biesenkamp-Uhe *et al.*, 2007; Karunakaran *et al.*, 2010; Reinhold *et al.*, 2011; Schautteet, De Clercq and Vanrompay 2011). However, vaccines based on humoral immunity alone are unlikely to efficiently protect against infections by intracellular pathogens (Seder and Hill 2000; Rodrigues *et al.*, 2003; Robinson and Amara 2005). Notably, pAPCs and their MHC-antigen presentation machinery are at the center of the initiation of immune responses by T cells and appear to be particularly important for the development of anti-chlamydial immunity (Karunakaran *et al.*, 2010). In our opinion, T cell vaccines that induce cellular immune responses, including activation of APCs and generation of long-lived T-cell memory, are holding the greatest promise for generating protective immunity against *Chlamydia* spp.

CONCLUSIONS AND OUTLOOK

Recent research has revealed a number of remarkable properties distinguishing *C. psittaci* from other members of the family *Chlamydiaceae*, which include

(i) a broad host range with preference for birds,
(ii) robust growth in cell culture and embryonated chicken eggs,
(iii) rapid and efficient entry into host cells, controlled by specific surface proteins and T3SS effectors,
(iv) efficient dissemination within the animal host causing systemic disease,
(v) the capability of causing a fulminant course of infection,
(vi) timely and strong upregulation of essential chlamydial genes in the face of the host immune response,
(vii) rigorous shutdown of *C. psittaci* gene expression in intracellular persistence *in vitro*,
(viii) inhibition of apoptosis as integral part of the *in vitro* persistence phenotype,
(ix) IncA and IncB protein-mediated utilization of host proteins to control cell survival and intracellular transport of inclusions in order to support bacterial growth in infected cells,
(x) pronounced physical attachment of bacterial inclusions to mitochondria to acquire eukaryotic substrates, and
(xi) a possible association with cancerous disease, such as ocular adnexal MALT lymphoma.

While these features appear to be relevant for evaluation of the pathogenic potential, it has to be noted that whole-genome analysis has not identified disease- or host-associated genetic markers to date, so that the underlying mechanisms still await exhaustive elucidation at the molecular level.

Although much has been learned about the developmental cycle of *C. psittaci* and other chlamydial species, several important questions regarding pathogen–host interaction are still open.

(i) How does chlamydial attachment and uptake exactly take place at the molecular level?
(ii) Is there a specific entry mechanism for each chlamydial species and its host cell counterpart?
(iii) What intracellular mechanism(s) enable(s) the pathogen to avoid fusion of inclusion and endo- or lysosomal compartments?
(iv) Which signal transduction pathways become activated at the various stages of the chlamydial developmental cycle?

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(v) Which molecular mechanisms control lipid supply to inclusions and release of chlamydiae from the host cell?

Recently developed murine and bovine infection models lend themselves as versatile tools to study both innate and adaptive immunity against bacterial infections. In addition, future projects using advanced cell biological and genomic approaches will help to answer these essential questions in order to unravel the remaining mysteries of chlamydial cell biology and infection.

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